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PDZK1: II. An anchoring site for the PKA-binding protein D-AKAP2 in renal proximal tubular cells

SERGE M. GISLER, CAVEH MADJDPOUR, DESA BACIC, SANDRA PRIBANIC, SUSAN S. TAYLOR, JÜRG BIBER, and HEINI MURER

Department of Physiology, University of Zürich, Zürich, Switzerland; and Department of Chemistry and Biochemistry, Howard Hughes Medical Institute, University of California, San Diego, California

PDZK1: II. An anchoring site for the PKA-binding protein D-AKAP2 in renal proximal tubular cells.

Background. PDZK1, a multiple PDZ protein, was recently found to interact with the type IIa Na/P_i cotransporter (NaPi-IIa) in renal proximal tubular cells. In a preceding study, yeast two-hybrid screens using single PDZ domains of PDZK1 as baits were performed. Among the identified proteins, a C-terminal fragment of the dual-specific A-kinase anchoring protein 2 (D-AKAP2) was obtained by screening PDZ domain 4.

Methods. After its molecular cloning by means of RACE, the renal expression of D-AKAP2 was analyzed by real-time polymerase chain reaction (PCR) and immunohistochemistry. Protein interactions were characterized by overlays, pull-downs, and immunoprecipitations from transfected opossum kidney (OK) cells.

Results. Based on 5′-RACE and PDZK1 overlays of mouse kidney cortex separated by two-dimensional electrophoresis, it was suggested that the renal isoform of D-AKAP2 in mouse comprises 372 amino acids and exists as a protein of >40 kD. Immunohistochemistry and real-time PCR localized D-AKAP2 only to the subapical pole of proximal tubular cells in mouse kidney. In pull-down experiments, D-AKAP2 tightly bound PDZK1 as well as N^+/H^+ exchanger regulator factor (NHERF-1), but the latter with an apparent fourfold lower affinity. Similarly, His-tagged D-AKAP2 specifically retained PDZK1 from mouse kidney cortex homogenate. In addition, *myc*-tagged D-AKAP2 and HA-tagged PDZK1 co-immunoprecipitated from transfected OK cells.

Conclusion. We conclude that D-AKAP2 anchors protein kinase A (PKA) to PDZK1 and to a lesser extent to NHERF-1. Since PDZK1 and NHERF-1 both sequester NaPi-IIa to the apical membrane, D-AKAP2 may play an important role in the parathyroid hormone (PTH)-mediated regulation of NaPi-IIa by compartmentalization of PKA.

Renal proximal tubules reabsorb the majority of filtered phosphate ions [1, 2]. Targeted inactivation of the *Npt2* gene demonstrated that the type IIa Na^+-dependent phosphate cotransporter (NaPi-IIa), which is localized at the brush borders of proximal tubular cells, plays a major role in this reabsorption process [3, 4]. To adjust the extent of proximal tubular reabsorption of inorganic phosphate (P_i) to the body needs, the abundance of NaPi-IIa cotransporters in the apical membrane is controlled via various hormones and metabolic factors [2]. Such an alteration of the number of apical NaPi-IIa is achieved (at constant rate of de novo synthesis) by regulated endocytosis, after which the internalized NaPi-IIa proteins are degraded in lysosomes [5–7].

Activation of several protein kinases has been found to control endocytosis of the type IIa Na/P_i cotransporter. Recent studies from opossum kidney (OK) cells from in vitro perfused murine proximal tubules and from in vitro incubated kidney slices have revealed an intracellular regulatory network, comprising protein kinase A (PKA), protein kinase C (PKC), protein kinase G (PKG) and mitogen-activated protein kinase (MAPK), that is implicated in the regulation of NaPi-IIa [2, 8–12]. It thus appears conceivable that specific regulation of the NaPi-IIa cotransporter requires a spatial organization of the protein kinases involved.

PDZK1 and N^+/H^+ exchanger regulator factor (NHERF-1) are multivalent PDZ-domain containing proteins expressed in the apical pole of various epithelial cells [13–16]. In proximal tubular cells, they are both important for a proper apical sorting and positioning of the type IIa Na/P_i cotransporter [15, 17–19]. Besides its implication in sorting and scaffolding of various other membrane and signaling proteins [20, 21], NHERF-1 also sequesters PKA via binding the ancillary actin-binding and A-kinase anchoring ERM protein ezrin [22]. The importance of this function of NHERF-1 was demonstrated in the case of the Na^+/H^+ exchanger 3 (NHE-3), where compartmentalization of PKA by NHERF-1 confers cyclic adenosine monophosphate (cAMP)-mediated down-regulation of NHE-3 activity through phosphory-

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lation of its intracellular C-terminus [23–25]. However, it has not been described so far if PDZK1 also acts as an indirect anchor for protein kinases known to be involved in the endocytosis of NaPi-IIa.

A yeast two-hybrid screen with the single PDZ domain four of PDZK1 as bait revealed the dual PKA-binding protein (D-AKAP2) [26, 27] to interact with PDZK1 (see preceding paper, [28]). In the present study, we investigated the interaction of PDZK1 with D-AKAP2 in more detail and describe D-AKAP2 as a proximal tubular, apical/subapical protein. Our data suggest that PDZK1, besides NHERF-1, provides a new anchor for PKA via binding to D-AKAP2 in proximal tubular cells.

METHODS

Cloning of renal D-AKAP2

Mouse kidney cDNA derived from full-length capped mRNA (Ambion, Huntingdon, UK) was used for 5′-rapid amplification of the N-terminal fragment of D-AKAP2. Polymerase chain reaction (PCR) (35 cycles, annealing temperature 55°C, elongation time 2 minutes) was performed with TITANIUM *Taq* polymerase (Clontech, Heidelberg, Germany) using 5′-TTTGGCAGC AAGCTGAGACTGGA and 5′-GGCTGACTCACA GAAGAGGATATC as antisense and nested-antisense primers, respectively. The nested amplicon (330 bp) was T/A cloned in pGEM-T Eeasy (Promega, Madison, WI, USA) and sequenced (Microsynth GmbH, Balgach, Switzerland). An identical fragment was obtained from cDNA derived from isolated mouse kidney polyA$^+$ RNA (SMART RACE, Clontech).

Expression of D-AKAP2 in oocytes of *Xenopus laevis*

The yeast clone D16 coding for the last 299 C-terminal amino acids of D-AKAP2 was introduced into the plasmid pSDEasy, which was modified to provide a c-*myc* tag at the 5′-end (N-terminus) of inserted sequences. Oocytes were injected with 10 ng of cRNA and processed for Western blotting after 3 days of incubation as described [29]. Expression of D-AKAP2 was detected either by a monoclonal anti-*c-myc* antibody (Molecular Probes, Eugene, OR, USA) or by a custom-made rabbit antiserum raised against a synthetic C-terminal peptide of D-AKAP2.

Stripping of membranes from mouse kidney cortex homogenate (MSM)

Kidney cortex slices from two mice (NMRI, 8 weeks old) were homogenized (Potter Elevehijem) in 2 mL of isolation buffer [5 mmol/L Tris-HCl, pH 7.1, 100 mmol/L mannitol, 1.5 mM ethyleneglycol tetraacetic acid (EGTA)] containing 1% protease inhibitors (P-8340) (Sigma Chemical Co., St. Louis, MO, USA) and centrifuged at 900 × *g* for 10 minutes at 4°C. The supernatant was diluted into 10 mL of ice-cold stripping buffer [15 mmol/L NaOH, 2 mmol/L Na_2 ethylenediaminetetraacetic acid (EDTA), and 1 mmol/L dithiothreitol (DTT), pH 11.0]. After five strokes in a potter (on ice) and centrifugation at 60,000 × *g* for 35 minutes at 4°C, the supernatant was neutralized to pH 7.4 by gently mingling 320 μL of 1 mol/L N-2-hydroxyethylpiperazine-N′-2ethanesulfonic acid (HEPES) in a glass beaker at 4°C. Proteins were concentrated by precipitation with methanol/chloroform following the procedure of Wessel and Flugge [30]. Dried pellet was resuspended in 3.5 mL of rehydration buffer RBN [7 mol/L urea, 2 mol/L thiourea, 4% (vol/vol) CHAPS, 0.5% (vol/vol) Triton X-100, 0.2 mol/L NDSB-256 (Calbiochem, San Diego, CA, USA), 40 mmol/L Tris base, 50 mmol/L DTT, 0.5% (vol/vol) IPG buffer, trace of Bromphenol blue, and 1% Sigma protease inhibitor cocktail P-8340] by means of mild sonication (30 seconds). After centrifugation at room temperature for 2 minutes at 25,000 × *g* and determination of the total protein content (Bradford, BioRad, Reinach, Switzerland), samples were kept at –80°C until use.

Two-dimensional gel electrophoresis

Mouse kidney cortex homogenate stripped of membranes (MSM) in RBN (0.25 μg/μL, see above) was kept at room temperature for 30 minutes, sonicated (30 seconds, pulsed), and cleared by centrifugation (16,000 × *g*, 10 minutes at room temperature). Totally, 20 μg (80 μL) of proteins were loaded by conventional sample cup application at the anodic site of a rehydrated Immobiline DryStrip (7 cm, pH 3-10) (Pharmacia, Duebendorf, Switzerland). Isoelectric focusing (IPGphor, Pharmacia) was conducted at 20°C with a voltage profile that gradually ramped from 500 V in 1 minute to 4000 V in 1.5 hours. The run was extended to 6500 Vh at 8000 V.

For separation in the second dimension, proteins were reduced and alkylated by incubating the strips twice for 15 minutes in 5 mL of equilibration buffer (EB) [50 mmol/L Tris-HCl, pH 8.8, 6 mol/L urea, 30% (vol/vol) glycerol, 2% (wt/vol) sodium dodecyl sulfate (SDS), and trace of Bromphenol blue] that was first supplemented with 2% (wt/vol) DTT and then with 2.5% (wt/vol) iodoacetamide. Strips were sealed with 0.5% low-melting agarose in 25 mmol/L Tris base, 190 mmol/L glycine, and 0.1% SDS on the top of a 10% SDS-polyacrylamide gel electrophoresis (PAGE) (with stacker) and run together with marker proteins (BioRad) containing 20 μg of kidney homogenate.

Blot overlay assay with radiolabeled glutathione-S-transferase (GST)/Ras-PDZK1

A total of 20 μg of mouse kidney cortex homogenate stripped of membranes (MSM) were separated in two dimensions (see above) and processed for overlay with GST/Ras-PDZK1. Purification of the probe GST/Ras-

PDZK1, labeling with [γ-^{32}P] guanosine triphosphate (GTP) and conditions of the overlay procedure are described in the preceding paper [28].

Pull-down experiments

D-AKAP2 and the cytosolic C-terminus (full-length or truncated for TRL) of NaPi-IIa were purified as N-terminal His-tagged fusions, immobilized on Ni-NTA beads, incubated with radiolabeled GST/Ras-PDZK1 or GST/Ras-NHERF-1 constructs and processed according to the His fusion pull-down in the preceding paper [28]. Pull-downs were performed with 500 μg of unstripped mouse kidney cortex homogenate (MCH).

OK-cell culture, transfections, immunoprecipitations, and immunocytochemistry

D-AKAP2 or PDZK1 were N-terminal tagged by cloning in *Xho*I/*Hin*dIII sites of *myc*-pcDNA3.1/Hygro(−) or in *Xho*I/*Eco*RV sites of HA-pcDNA3.1/Hygro(−), respectively. Using 5 μg of *myc*/D-AKAP2 and 5 μg of HA/PDZK1 in the presence of 45 μL Lipofectamine (Gibco/BRL, Basel, Switzerland), OK cells, grown to subconfluency in a 10 cm dish (Nunc, Neerijse, Belgium), were co-transfected overnight as described [18]. Reaching confluency, cells from one dish were washed three times with Tris-buffered saline (TBS) (50 mmol/L Tris-HCl, pH 8.0, and 150 mmol/L NaCl) and lysed at 4°C with 1 mL of TBS containing 1% Igepal CA-630 and 1% protease inhibitors (Sigma P-8340). Lysates were passed several times through a 23-G needle and centrifuged for 5 minutes at 4000 × *g*. To reduce nonspecific binding, supernatants were pretreated with 50 μL of protein-A/G agarose beads slurry (Oncogene, San Diego, CA, USA) for 60 minutes. After removal of the beads at 10,000 × *g* for 3 minutes, samples were incubated overnight either with 1 μL of monoclonal anti-*myc* (Invitrogen, Basel, Switzerland) or anti-HA antibodies (Sigma Chemical Co.) in the presence of fresh agarose beads (50 μL). To recover immunoadsorbed proteins, collected beads (1 minute at 10,000 × *g*) were washed three times with lysis buffer (0.5 mL). Finally, proteins were denatured at 95°C for 2 minutes with 50 μL 2 × SDS-PAGE loading buffer and 50 μg thereof were loaded on a 10% SDS-PAGE.

Confluent double-transfected cells were analyzed by confocal microscopy using a Leica TCSSP laser scan microscope (Leica, Wetzlar, Germany) equipped with a 40× oil-immersion objective and an IMARIS program.

Immunohistochemistry

The distribution pattern of D-AKAP2 in mouse kidney cortex was investigated by indirect immunofluorescence as described [10]. The primary antibodies were as follows: rabbit polyclonal antimouse PDZK1 (1:500) [15], rabbit or guinea pig polyclonal antimouse D-AKAP2 (1:1000), and rabbit polyclonal anti-rat NaPi-IIa (1:500) [3].

Laser microdissection and real-time PCR

Male NMRI mice at the age of 8 weeks were sacrificed and kidneys were immediately snap frozen in liquid nitrogen. Cryosections (6 μm) were mounted on polyethylene membrane slides (Molecular Machines & Industries, Glattbrugg, Switzerland) and processed as described [Madjdpour C et al, submitted for publication]. Laser microdissection was performed on kidney slices obtained from three different animals by an inverting microscope with motorized scanning stage that was equipped with a solid-state laser in the ultraviolet region (Molecular Machines & Industries). Both S_1 and S_3 segments of superficial nephrons were identified by phase-contrast microscopy. A total area corresponding to 100,000 μm^2 (± 1%) was microdissected for each sample.

RNA was extracted using the Absolutely RNA™ Nanoprep Kit (Stratagene, Amsterdam, The Netherlands). First-strand cDNA from total RNA was synthesized in a reaction volume of 50 μL with TaqMan Reverse Transcription Reagents (Applied Biosystems, Rotkreuz, Switzerland) in the presence of random hexamers according to the manufacturer's instructions.

Relative quantization of D-AKAP2 mRNA was achieved by means of an ABI PRISM 7700 Sequence Detection System (Applied Biosystems) with β-actin as an internal standard. The sequences of TaqMan probes (Biosearch Technologies, Novato, CA, USA) and primers (Microsynth) were as follows: 5′-(6-FAM)-CCATGA GAAACGACATCATCGCAAAGATTT-(BHQ-1)-3′ (probe), 5′-CTGCTAAGCCAATACCAATTACA GAA-3′ (forward), 5′-AGTTGGGATCCACCTGT CCA-3′ (reverse) for D-AKAP2 and 5′-(6-FAM)-CCAT GAAGATCAAGATCATTGCTCCTCCT-(BHQ-1)-3′ (probe), 5′-GACAGGATGCAGAAGGAGATTA CTG-3′ (forward), 5′-CCACCGATCCACACAGAGT ACTT-3′ (reverse) for β-actin. TaqMan probes were set across exon-exon boundaries to exclude any amplification of genomic DNA. PCR reactions in a volume of 25 μL, containing 900 nmol/L gene-specific primers and 250 nmol/L probes, were performed using TaqMan Universal PCR Master Mix (Applied Biosystems). After incubation with uracil-*N*-glycosylase (2 minutes at 50°C) and activation of AmpliTaq Gold DNA polymerase (10 minutes at 95°C), the samples were amplified by 45 cycles at 95°C for 15 seconds and 60°C for 1 minute. Relative quantification and normalization were attained through the software Q-Gene [31]. Standard curves for D-AKAP2 and β-actin were generated based on total kidney mouse RNA.

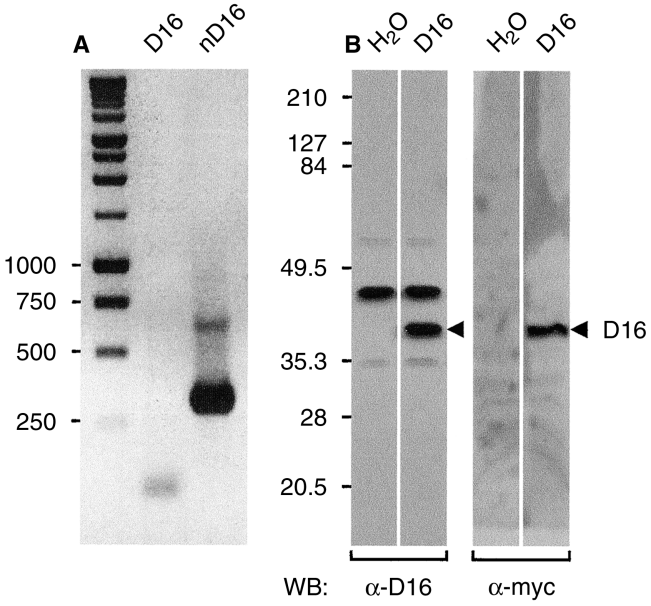

Fig. 1. Identification of renal dual-specific A-kinase anchoring protein 2 (D-AKAP2). (*A*) 5′-RACE. Two different cDNA templates and a combination of nine specific primers were used to amplify the 5′-missing sequence of the partial clone D16. Amplicons, obtained before (D16) and after (nD16) nested polymerase chain reaction (PCR), were resolved by agarose gel electrophoresis. (*B*) Immunodetection of D-AKAP2. Oocytes were injected with water or *myc*-tagged D16 comprising 299 residues of the C-terminal portion of D-AKAP2. After separation (30 μg of lysate), D16 was immunodetected with either an anti-*myc* antibody or a polyclonal antiserum raised against the very C-terminal peptide of D-AKAP2. WB is Western blot.

RESULTS

Identification of D-AKAP2

In the preceding paper [28], we described yeast two-hybrid screens against a cDNA library derived from adult mouse kidneys with single PDZ domains of PDZK1 as targets. Among the different proteins obtained from the screen with the fourth domain (PDZ 4) of PDZK1 as bait, one clone, designated D16, encoded for the 299 C-terminal amino acids of D-AKAP2 [26].

Based on a plaque screen of a cDNA library derived from an adult mouse testis, the full-length D-AKAP2 was originally reported to consist of 372 amino acid residues [26]. Later on, an isoform comprising 662 residues was identified in human brain [27]. To resolve this apparent discrepancy, we first identified the missing sequence information toward the 5′-end of the partial clone D16 by means of 5′-RACE. A group of nine gene-specific primers and two different sources of cDNA templates from mouse kidneys were used. After nested PCR, the same amplicon as shown in Figure 1A was obtained with both templates although with different combinations of primers. In all experiments, no larger fragments could be amplified. Based on the size and the sequence of the amplified 5′-fragment, we concluded that D-AKAP2 in kidneys of adult mice consists of 372 amino acids as initially reported for testis [26].

Renal distribution of D-AKAP2

The occurrence of D-AKAP2 in mouse tissues has recently been investigated at both mRNA and protein levels [26, 27]. These studies demonstrated that D-AKAP2 is expressed rather ubiquitously, with strongest expression of its mRNA in lung, testis and kidney. However, the cellular location of D-AKAP2 in renal tissue has not been investigated so far. To examine the distribution of D-AKAP2 in mouse kidney, a polyclonal antibody was raised against a C-terminal synthetic peptide of mouse D-AKAP2. The specificity of this antiserum was tested by Western blots performed with lysates of *Xenopus laevis* oocytes that were injected with cRNA coding for the *myc*-tagged original clone D16 (299 amino acids) or with water. As illustrated in Figure 1B, a band of 40 kD was immunodetected with an antibody against c-*myc* in *myc*/D16-cRNA–injected oocytes, but not in control oocytes. In *myc*/D16 oocytes, the same band was also observed using the antiserum raised against the C-terminus of D-AKAP2.

In cryostat sections of mouse kidney cortex, D-AKAP2–related immunofluorescence was detected only in proximal tubular cells, with highest intensity in S_1 segments of superficial and deep nephrons (Fig. 2A and B). This staining was completely attenuated after preincubation of the antiserum with the antigenic peptide indicating specificity of the applied antiserum (Fig. 2C). An identical distribution of D-AKAP2 was observed with antibodies provided by Wang et al [27] (data not shown). Consecutive cryosections were also stained for the type IIa Na/P_i cotransporter and the PDZ protein PDZK1, both of which have been localized in proximal tubular cells [3, 14, 15]. As delineated in Figure 2A and B, NaPi-IIa and D-AKAP2 exhibited a similar intranephronal distribution pattern along the proximal tubule ($S_1 > S_2 > S_3$), whereas the PDZK1-mediated immunostaining along this proximal tubular axis was of uniform intensity. We were unable to discern any internephronal heterogeneity (superficial versus juxtamedullar) of the three proteins. When D-AKAP2 was co-stained with NaPi-IIa, higher magnification revealed that D-AKAP2 and NaPi-IIa did not overlap completely (Fig. 2D). NaPi-IIa was entirely associated with the brush borders, whereas D-AKAP2 was more confined to the subapical compartment and rather weakly localized at the base of the microvilli. As a consequence of their strict localization in the brush borders, co-stains of NaPi-IIa and PDZK1 with D-AKAP2 were coincident (not shown).

In addition, the expression of D-AKAP2 mRNA was analyzed by real-time PCR after laser microdissection of superficial S_1 and S_3 proximal tubular segments (Fig. 3). When the relative amounts of D-AKAP2 mRNA

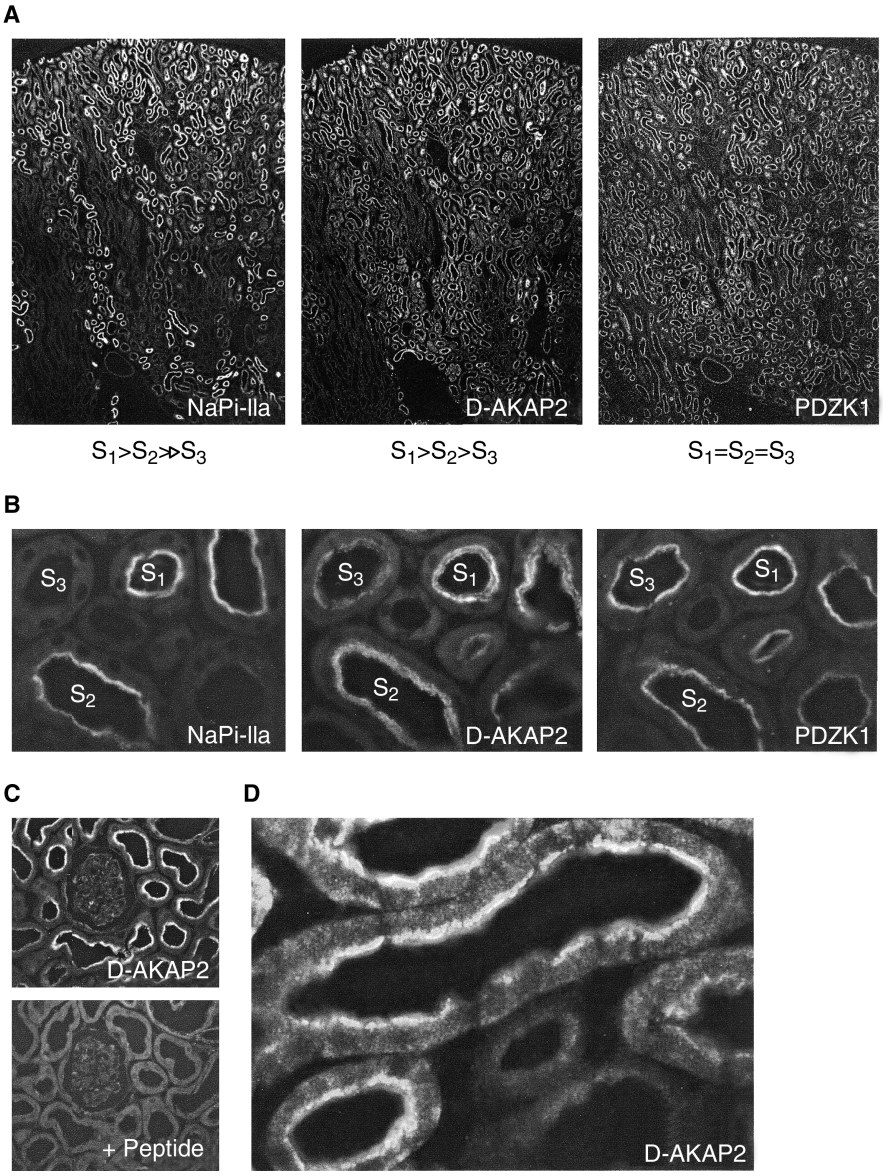

Fig. 2. Immunohistochemical localization of dual-specific A-kinase anchoring protein 2 (D-AKAP2) in mouse kidney. Antibodies directed against type IIa Na/Pi cotransporter (NaPi-IIa), D-AKAP2, and PDZK1 were applied to consecutive cryosections of mouse kidney and analyzed by fluorescent microscopy (*A*). All proteins are only expressed in proximal tubular cells, yet with different intranephronal abundance. At higher magnification (*B*), NaPi-IIa, PDZK1, and D-AKAP2 are only confined to the apical site of these cells. To control for the specificity of the anti-D-AKAP2 immunostain, the antiserum was pretreated with the synthetic antigenic peptide before application (*C*). The proximal segments S_1, S_2, and S_3 are typified. Co-staining for NaPi-IIa and D-AKAP2 showed overlap of the two proteins at the base of microvilli and significant overlap of D-AKAP2 in the subapical compartment (*D*).

were compared to β-actin mRNA in microdissected samples obtained from kidneys of three different mice, an approximately threefold higher abundance of D-AKAP2–related mRNA was detected in S_1 segments compared to S_3 segments. Therefore, the relative mRNA quantities and the immunohistochemical intensities of D-AKAP2 in the S_1 and S_3 segments entirely correlated.

In vitro binding studies

Initial blot overlays and pull-downs with recombinant proteins suggested that D-AKAP2 can interact with PDZK1 outside the yeast two-hybrid system (see Figs. 2A and 3 of preceding paper [28]). To corroborate these findings, four different assays were applied. First, a soluble fraction of mouse kidney homogenate was separated by two-dimensional electrophoresis and overlaid with [^{32}P]GTP-labeled GST/Ras-PDZK1. As illustrated (Fig. 4B), only a few spots from the total protein stain (Fig. 4C) were detected by labeled GST/Ras-PDZK1 in the range of 30 to 50 kD. One of these spots, at the predicted size of D-AKAP2 (>40 kD) and at an isoelectric point of pH 5.5, was also immunostained by the anti-D-AKAP2 antibody (Fig. 4D). Because no spots were present when [^{32}P]GTP-GST/Ras was used as the probe (Fig. 4A), the radiolabeling of proteins on the blot was due to the specific binding of PDKZ1 itself. Thus, recombinant PDZK1 can bind endogenously expressed D-AKAP2 of >40 kD. The size of >40 kD further accords with the existence of a renal isoform of D-AKAP2 that contains 372 amino acids.

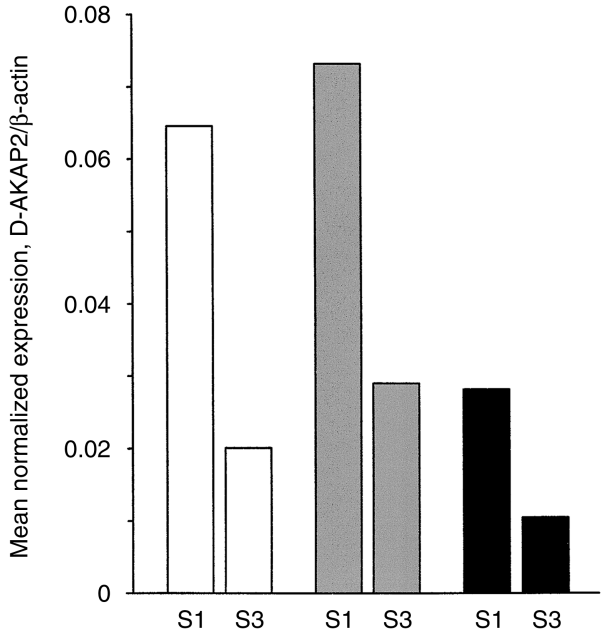

Fig. 3. Proximal tubular expression of dual-specific A-kinase anchoring protein 2 (D-AKAP2) mRNA. Proximal tubular segments S_1 and S_3 of superficial nephrons from three kidneys (white, gray, and black bars) of adult mice were isolated by laser-assisted microdissection. Relative quantification of D-AKAP2-related mRNA was determined by real-time polymerase chain reaction (PCR) with β-actin as an internal standard. For each sample, an identical area was analyzed.

Second, an isotopic pull-down assay was performed. On account of the weak interaction of NHERF-1 with D-AKAP2, that was observed in the blot overlay of the preceding paper (see Fig. 2B of preceding paper [28]), NHERF-1 was included as comparison. In this approach, radiolabeled GST/Ras-PDZK1 and GST/Ras-NHERF-1 were incubated with an immobilized His/soluble protein D (SP^D)-tagged fusion to D-AKAP2 or the C-terminus of NaPi-IIa as a control. Bound PDZK1 or NHERF-1 was determined by scintillation counting. As illustrated in Figure 5A, much higher amounts of PDZK1 than of NHERF-1 were retained by D-AKAP2 indicating strong interaction with PDZK1. By comparison, the apparent affinity due to the interaction of NHERF-1 with D-AKAP2 was approximately fourfold lower and was comparable to the interaction of either PDZK1 or NHERF-1 with the C-terminus of NaPi-IIa. To assess for specificity of this assay, the fusion partner His/SP^D alone and the truncated C-terminus (minus TRL) of NaPi-IIa were exposed to the labeled fusion proteins. In both cases, retention of radiolabeled fusion proteins was negligible or very low. Likewise, the pull-downs of radiolabeled GST/Ras alone were negative as well.

Next, we examined if endogenously expressed PDZK1 can be pulled down from a mouse kidney homogenate by His/SP^D-tagged D-AKAP2. After extensive washing, protein complexes were eluted by imidazole and subjected to immunoblotting against PDZK1 (Fig. 5B). Both the pull-down with His/SP^D-D-AKAP2 (but not with His/SP^D alone) and the homogenate caused identical PDZK1-immunoreactive bands at 70 kD, inferring that PDZK1 was specifically precipitated by His/SP^D-D-AKAP2.

Finally, the association of PDZK1 with D-AKAP2 was investigated by immunoprecipitation after co-transfection of OK cells with HA-tagged PDZK1 and *myc*-tagged D-AKAP2 (Fig. 6). In these cells, HA/PDZK1 was targeted to the (sub)apical membrane, whereas the distribution of *myc*/D-AKAP2 was not restricted and therefore merely co-localized with PDZK1 in a subapical rim (Fig. 6B). In an immunoprecipitate against the HA-tag, *myc*-tagged D-AKAP2 was withdrawn only in double-transfected OK cells but not in OK cells that were not transfected (Fig. 6A) or solely transfected with *myc*-tagged D-AKAP2 alone (not shown). Similarly, HA/PDZK1 was co-immunoprecipitated with anti-*myc* antibodies only after co-transfection (not shown).

DISCUSSION

Our study revealed the expression of a new protein, denoted D-AKAP2, in the renal proximal tubular cells of adult mice. D-AKAP2 has been described before as a putative dual-specific PKA-anchoring protein in testis and brain tissue [26, 27]. In testis, D-AKAP2 was initially cloned as a multifunctional protein of 372 amino acids containing an N-terminal domain for G protein signaling (RGS domain), a C-terminal anchoring helix for the regulatory subunits RI and II of PKA and a PDZ-binding motif at the extreme end of the C-terminus [32]. Thus, D-AKAP2 could provide a link between two major components of the cAMP-signaling transduction pathway. First, it might recruit PKA into a physically and functionally distinct signaling unit. Second, the RGS domain might drive heterotrimeric G proteins into their inactive guanosine diphosphate (GDP)-bound state by accelerating the GTPase activity of G-protein α subunits [26, 33, 34]. Besides testis, a longer form of D-AKAP2 comprising 662 amino acids, which exhibits an additional RGS domain, was recently reported in human brain [27]. Since we could not identify such a longer protein in mouse kidney, we suggest that D-AKAP2 of 372 amino acids is the only form expressed in murine adult kidney and could most likely represent a splice variant.

Originally, we identified D-AKAP2 in a yeast two-hybrid screen with the fourth PDZ domain of PDZK1 as a target (see preceding paper [28]). Independent of the yeast two-hybrid system, a strong interaction of D-AKAP2 with PDZK1 was also corroborated by means of different in vitro assays that were performed in the previous (Figs. 2 and 3 of preceding paper [28]) and the present (Figs. 4 to 6) report. We anticipate that this PDZ-mediated interaction is probably direct via the C-terminus of D-AKAP2, as its very end (STKL) represents

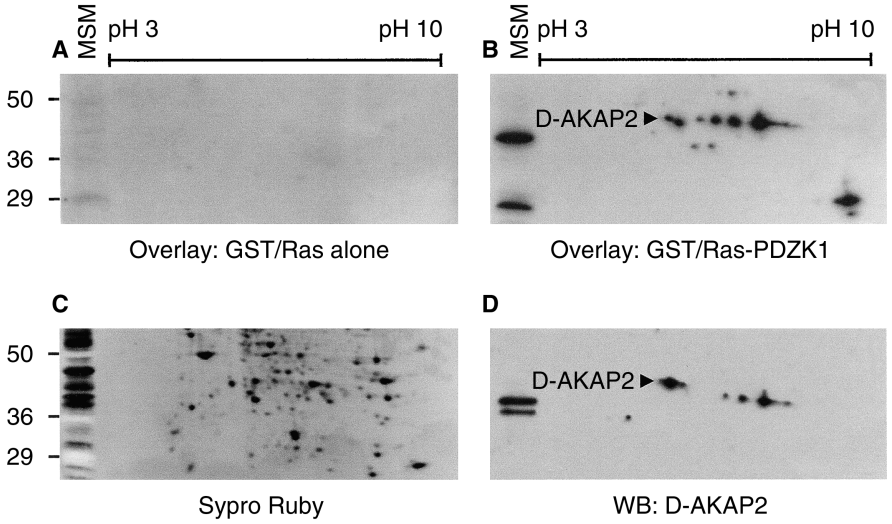

Fig. 4. Blot overlay. Two-dimensional electrophoresis of mouse kidney cortex homogenate stripped of membranes (20 μg) was performed as described in the **Methods** section. The blots of two parallel runs were overlaid either with 10 μg of radiolabeled glutathione-S-transferase (GST)/Ras (*A*) or GST/Ras-PDZK1 (*B*) and autoradiographed. Total protein was detected by staining with Sypro (*C*). After autoradiography, the blot (B) was stripped with ethylenediaminetetraacetic acid/sodium dodecyl sulfate (EDTA/SDS) and reprobed with the antiserum against D-AKAP2 (*D*). Due to differences of protein entries into the gel, proteins from the strips are retarded in migration compared to the standards. MSM is mouse kidney cortex homogenate stripped of membranes; WB is Western blot.

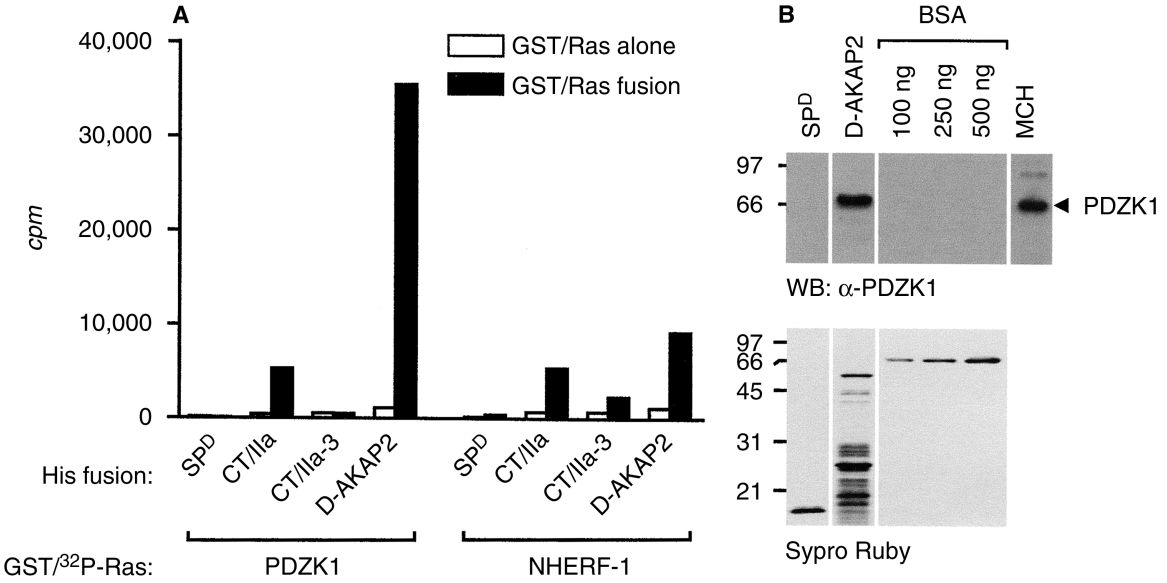

Fig. 5. Pull-down experiments. (*A*) Isotopic pull-downs. Full-length (CT/IIa) or truncated (C-terminal TRL omitted; CT/IIa-3) C-terminus of type IIa Na/Pi cotransporter (NaPi-IIa) and dual-specific A-kinase anchoring protein 2 (D-AKAP2) were batch-bound (~15 μg each) to Ni-NTA magnetic agarose beads over the His-tag of the N-terminal fusion partner soluble protein D (SP^D). The samples were incubated with 1 μg of [^{32}P] guanosine triphosphate (GTP)-labeled glutathione-S-transferase (GST)/Ras (□), GST/Ras-PDZK1, or GST/Ras- N^+/H^+ exchanger regulator factor (NHERF-1) (■) for 2 hours at 4°C. After intense washing, proteins were released from the beads with imidazole and retained radioactivity in the eluates was determined by liquid scintillation. (*B*) Pull-downs from murine kidney cortex homogenate. His-tagged SP^D alone or His-tagged SP^D-D-AKAP2 (15 μg each) was incubated with 500 μg kidney cortex homogenate for 2 hours at 4°C, thereafter washed, eluted, and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After blotting, equal loading was confessed by Sypro Ruby. Precipitated PDZK1 was detected by a Western blot (WB). MCH is mouse kidney cortex homogenate; BSA is bovine serum albumin.

a typical class I PDZ-binding motif [35]. In addition to PDZK1, our results also provide evidence that D-AKAP2 can bind NHERF-1, albeit not as strongly as with PDZK1.

In kidney, D-AKAP2 is localized only in the proximal tubular cells and partially overlapped with PDZK1 (Fig. 2) and NHERF-1 (not shown) at the base of microvilli. In these cells, both PDZ proteins, PDZK1 and NHERF-1, assemble a submembranous macromolecular complex, in which membrane proteins, such as NaPi-IIa and NHE-3, are clustered (see preceding paper [28], references [15, 20]). It will be a challenge in the future to elucidate the impact of the PDZ proteins in trafficking D-AKAP2 to close proximity with its targets, such as NaPi-IIa and NHE-3.

What could be the physiologic role of D-AKAP2 in

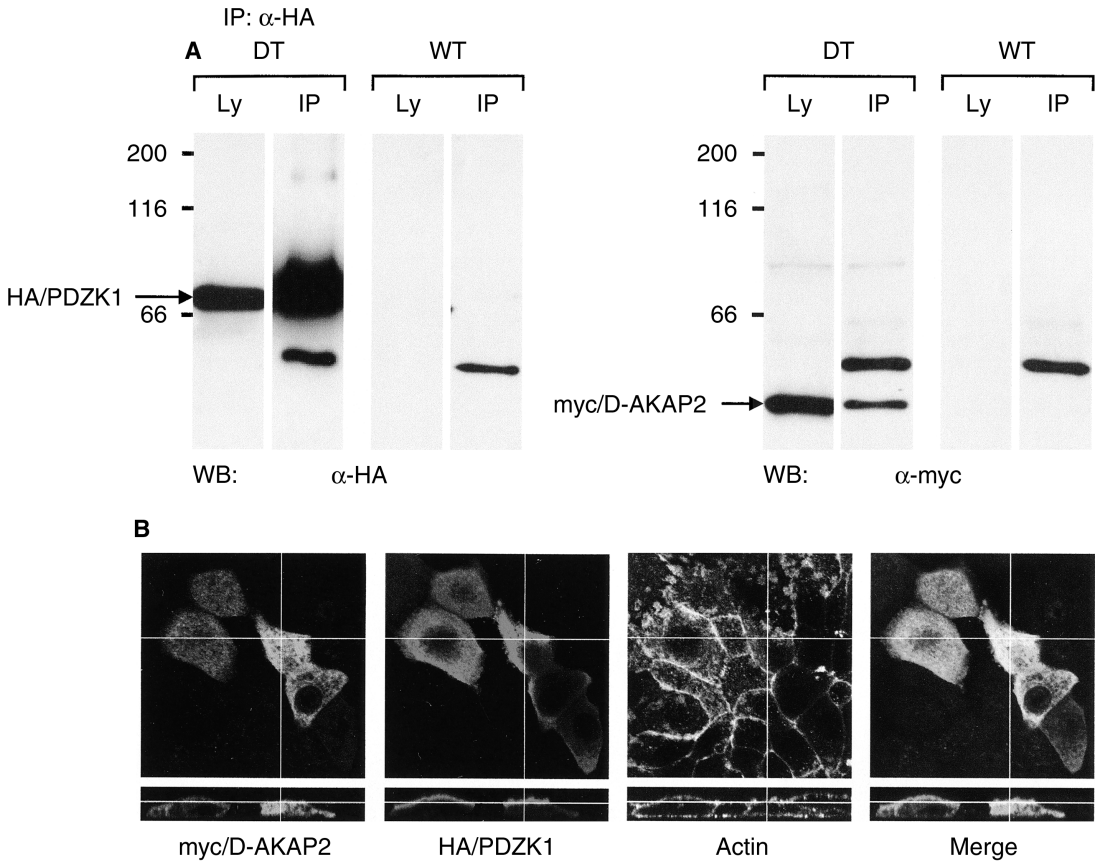

Fig. 6. Co-immunoprecipitations of dual-specific A-kinase anchoring protein 2 (D-AKAP2). (*A*) Immunoprecipitation of HA/PDZK1. Opossum kidney (OK) cells were co-transfected with HA/PDZK1 and *myc*/D-AKAP2. Lysates, obtained from untransfected (WT) and double-transfected (DT) cells, were subjected to immunoprecipitations against the HA-tag. Samples before (Ly) and after immunoprecipitation (IP) were separated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotted with anti-HA or anti-*myc* antibodies. When precipitated with *myc*-antibodies, HA/PDZK1 also co-immunoprecipitated (not shown). WB is Western blot. (*B*) Immunocytochemistry. Expression of HA/PDZK1 and *myc*/D-AKAP2 in OK cells was analyzed by confocal microscopy. Squares represent apical focal planes and rectangles confocal cross sections. A co-localization is reflected by a yellow signal (not shown) of the merged composite. In contrast to the apical constraint of HA/PDZK1, the localization of *myc*/D-AKAP2 in OK cells was not polarized. The actin stain evinces the integrity of the cell.

the regulation of NaPi-IIa? By triggering the PKA- and PKC-dependent pathways, parathyroid hormone (PTH) leads to internalization of NaPi-IIa in the apical membrane of proximal tubular cells [2]. Similarly, the activity of NHE-3 is decreased by phosphorylation and internalization, whereby phosphorylation through PKA was shown to be dependent on NHERF-1 [23, 24, 36]. So far, proteins implicated in down-regulation of NaPi-IIa after stimulation with PTH are still not known. Although its physiologic function is still an enigma, D-AKAP2 could represent one such candidate-signaling factor for PTH-mediated regulation of NaPi-IIa by providing an anchoring site for PKA in the brush borders of proximal tubular cells. As D-AKAP2 is mainly localized to the subapical compartment, a role either in endocytosis of NaPi-IIa at microvillar clefts or in the sorting of NaPi-IIa from the subapical compartment to the lysosomes is implied.

We postulate that PDZK1 and NHERF-1 could fulfill an intriguing pivotal role by tethering together NaPi-IIa, NHE-3, and D-AKAP2 at a highly specialized region of the plasma membrane. As PDZK1 also binds NHERF-1 [28] and the latter the receptor for parathyroid hormone (PTH1R) [37], NaPi-IIa and NHE-3 are clustered with most of the signaling molecules responsible for their PTH-mediated internalization. Because many of the downstream signaling molecules are used in other cellular pathways, this molecular scaffold enhances fidelity and speed of the PTH response by tethering the PKA pathway to NaPi-IIa (or NHE-3).

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Reprint requests to Dr. Jürg Biber, Institute of Physiology, University of Zürich, Winterthurerstrasse 190, CH-8057 Zürich, Switzerland. E-mail: JuergBiber@access.unizh.ch

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